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## WT1 MONOCLONAL ANTIBODIES AND METHODS OF USE THEREFOR

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Field of the Invention

This invention relates generally to the field of detecting, monitoring and diagnosing malignancies characterized by expression of the Wilms' tumor 1 antigen.

Background of the Invention

The Wilms' tumor (wt1) gene encodes a protein referred to as WT1 which is expressed in the nucleus of certain cells and possesses the structural features of a DNA binding transcription factor. As illustrated in Fig. 1 below, the WT1 protein is a 429 amino acid protein [SEQ ID NO:4] which contains four contiguous zinc fingers at the carboxyl-terminus, and a glutamine- and proline-rich region at the amino-terminus. The amino-terminal region of WT1 protein mediates transcriptional suppression or activation in transient transfection assays [Madden et al, Science, 253:1550-1553 (1991); Maheswaran et al, Proc. Natl. Acad. Sci. USA, 90:5100-5104 (1993); S. L. Madden et al, Oncogene, 8:1713-1720 (1993)]. Splice variants of WT1 can produce the protein with a 17 amino acid insert at amino acid 249 and/or a 3 amino acid insert at amino acid 390.

The wt1 gene encoding WT1 protein is located on chromosome 11p13 and has been found to be mutated or deleted in a subset of hereditary and sporadic Wilms' tumors. Recently, high levels of wt1 expression were reported in a variety of tumors such as ovarian carcinomas [Bruenig et al, Cancer Invest., 11:393-399

(1993)], prostate cancer, mesotheliomas [Park et al, cited above], and leukemias [Miwa et al, Leukemia, 6:405-409 (1992), Miyagi et al, Leukemia, 7:970-977 (1992)].

Diagnostic methods for the ovarian carcinomas, mesotheliomas, and leukemias referred to above are based primarily on clinical attributes and histology of tumor specimens. These methods may at times not distinguish between closely related diseases and may lead to inappropriate treatments of patients. For example, in addition to the presence of many histological variants of malignant mesothelioma, there are other lesions that can affect the pleural surface and present a clinical and histological picture quite similar to malignant mesothelioma [R. J. Pisani et al, Mayo Clin. Proc., 63:1234-1244 (1988)]. Additional relatively specific molecular markers that clearly distinguish between clinically similar lesions for malignant mesotheliomas as well as the other cancers would thus be a valuable clinico-pathological tool which will permit a precise diagnosis. This is important since treatment protocols and prognosis for such conditions vary significantly.

Currently available diagnostic tools include rabbit polyclonal antibodies for WT1 protein known in the art. Morris et al, Oncogene, 6:2339-2348 (1991) describe two such antibodies which recognize amino acid fragments spanning amino acids 294-429 of SEQ ID NO:4 and amino acids 85-173 of SEQ ID NO:4, respectively, of the WT1 protein. Another rabbit polyclonal antibody, which recognizes WT1 amino acids 275-429 of SEQ ID NO: 4 was described by Telerman et al, Oncogene, 7:2545-2548 (1992). Still other WT1 polyclonal antibodies are commercially available, e.g. the rabbit polyclonal antibody SC-192, which is available from Santa Cruz. However, while polyclonal antibodies in general are able to detect WT1 expression, they have disadvantages in

their potential for cross-reactivity with closely related proteins which share common domains with the WT1 protein. These polyclonal antibodies by their nature are likely to provide inconsistent results in antigen specificity and binding affinity studies and are not particularly desirable for diagnostic uses.

Additionally, a commercially available mouse monoclonal antibody, DG-10 (Applied BioTechnology) was raised to the zinc finger region of WT1 and is known to cross-react with the Egr1 proteins. Expression of Egr1 proteins is not limited to cells or tissues that express WT1 and is independently regulated from WT1 expression. Therefore, any antibodies raised to the zinc finger domain in the carboxyl terminus of WT1 may not be useful for selective detection of the WT1.

Another anti-WT1 mouse monoclonal antibody has been described by Mundlos et al, Development, 119:1329-1341 (1993). The Mundlos et al antibody is specific for a 17 amino acid sequence insert (See Fig. 1 below), i.e., a splice variant, that is present in only a subpopulation of the alternatively spliced WT1 mRNA messages.

Thus, there exists a need in the art for methods and compositions for detecting and differentially diagnosing conditions characterized by over-expression or inappropriate expression of WT1.

#### Summary of the Invention

In one aspect, the present invention provides a hybridoma cell line secreting a monoclonal antibody (MAb) specific for a protein antigen, referred to as WT1-6F [SEQ ID NO: 2], which contains amino acids 1-181 of WT1 [SEQ ID NO: 4]. One such cell line is an H2-secreting line, deposit designation ATCC No. 11598. Another cell line which is an embodiment of this aspect is the H7-secreting line, deposit designation ATCC No. 11599.

Still a third cell line is the HC17-secreting line, deposit designation ATCC No. 11600.

In another aspect, the present invention provides a monoclonal antibody produced by a cell line described above. Three such antibodies, designated H2, H7 and HC17 are described herein.

In yet another aspect, the invention provides the heavy chain and light chain variable region polypeptides of the MABs of the invention, and other fragments thereof, such as Fab fragments, F(ab)<sub>2</sub> fragments, Fv fragments and the like.

In still another aspect, the present invention provides methods of diagnosing malignancies characterized by over-production or inappropriate expression of WT1 protein. These methods involve screening biological samples with antibodies of the invention, described above.

In a further aspect, the present invention provides methods of monitoring treatment of conditions characterized by over-production or inappropriate expression or production of WT1 protein. One embodiment of such a method involves monitoring leukemia treatment, particularly determining the level of active leukemia following a treatment cycle.

In another aspect, the present invention provides methods for differentiating between malignancies characterized by over-production or inappropriate expression of WT1 protein and conditions having similar symptomatic profiles. One embodiment of such a method involves distinguishing between mesotheliomas and conditions characterized by inflammatory reactions.

In a still another aspect, the present invention provides kits useful for detecting, monitoring, and/or diagnosing a disease characterized by the expression of the Wilms' tumor antigen comprising a MAB raised against

the WT-6F antigen [SEQ ID NO: 2]. Desirably, the H2, H7, HC17 MAbs or a cocktail of these, is included in such a kit.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

#### Brief Description of the Drawings

Fig. 1 is a schematic diagram of the Wilms' tumor protein functional domains. The WT1 protein contains two discrete functional domains: the amino terminus contains a transcriptional regulator domain and the carboxy terminus contains a DNA binding domain with four C<sub>2</sub>H<sub>2</sub> zinc fingers. G/P refers to the glutamine- and proline-rich region at the amino-terminus; ZN refers to four contiguous zinc fingers at the carboxyl-terminus. Alternatively spliced transcripts of WT1 are produced which insert 17 amino acids, VAAGSSSSVKWTEGQSN, [SEQ ID NO: 7] (17AA) within the transcriptional regulatory domain (at amino acid 249 of SEQ ID NO: 4) or a tripeptide encoding the amino acid KTS within the DNA binding domain (at amino acid 390 of SEQ ID NO: 4) between zinc fingers 3 and 4. The significance of the alternatively spliced WT1 transcripts is not known.

Fig. 2 provides the nucleic acid and amino acid sequences of the WT-6F antigen [SEQ ID NOS: 1 and 2] in which amino acids 1-11 represent a histidine fusion protein to facilitate purification; amino acids 12-192 are amino acids 1-181 of the WT1 protein; and amino acids 193-210 of SEQ ID NO: 2 are vector sequences added during cloning.

Fig. 3 provides the nucleotide and amino acid sequences of the full length WT1 protein [SEQ ID NOS: 3 and 4]. The 3' non-coding sequence of the mRNA is omitted in this figure.

Detailed Description of the Invention

The present invention provides hybridomas secreting monoclonal antibodies (MAbs) specific for epitopes found in the amino terminal amino acids 1-181 of the Wilms' tumor (WT1) protein [SEQ ID NO: 4]. The MAbs of this invention are useful in identifying, monitoring and diagnosing conditions characterized by over-expression or inappropriate expression of the WT1 protein. The MAbs do not cross-react with the ubiquitous and closely related early growth response (Egr1) family of proteins which share approximately 50% homology within the DNA binding domain located in the carboxyl terminal amino acids 275-429 of WT1 [SEQ ID NO:4]. Therefore, when used in a diagnosis based on the detection of WT1 protein, the MAbs of this invention eliminate false positives currently produced in detection methods by the use of currently available WT1 antibodies which are specific for epitopes in the zinc finger domain of the protein.

20 I. Definitions

As used herein "functional fragment" is a partial complementarity determining region (CDR) sequence or partial heavy or light chain variable sequence of an antibody which retains the same antigen binding specificity and/or neutralizing ability as the antibody from which the fragment was derived.

A "condition characterized by over-expression or inappropriate expression of WT1" refers to a cancer or other abnormal physiological state which exhibits an increased level of expression of WT1 or exhibits expression of a mutant WT1 protein, or exhibits expression of WT1 protein where such expression should normally not occur. Such increased WT1 expression has been detected in cells derived from ovarian carcinomas, mesotheliomas, prostate cancer and leukemias.



Ordinarily, in normal tissues, WT1 protein is absent or present in such low levels that it cannot be detected using conventional techniques, such as northern blot hybridization or reverse transcriptase polymerase chain reaction (RT-PCR). In contrast to WT1 protein, when a patient exhibits a "condition characterized by over-expression or inappropriate expression of WT1" as defined herein, the presence of WT1 protein can be detected using the reagents of the invention and standard techniques, e.g. immunohistochemical procedures, including immunoblotting and immunofluorescence, Western blot analysis, and enzyme-linked immunosorbant assay (ELISA). The presence of WT1 mRNA in such patients can be detected using Northern blot analysis or RNA reverse transcription PCR techniques. Background levels of WT1 can be determined by measuring such levels in the tissues where WT1 is not normally expressed (as described above) in persons not afflicted with disease.

"CDRs" are defined as the complementarity determining region amino acid sequences of an antibody. CDRs are contained within the hypervariable regions of immunoglobulin heavy and light chains. CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived from donor antibody variable heavy and light chain sequences, and include functional fragments and analogs of the naturally occurring CDRs, which fragments and analogs also share or retain the same antigen binding specificity and/or neutralizing ability as the donor antibody from which they were derived.

By 'sharing the antigen binding specificity or neutralizing ability' is meant, for example, that although a given MAb may be characterized by a certain level of antigen affinity, and a CDR encoded by a nucleic acid sequence of the same MAb in an appropriate

structural environment may have a lower or higher affinity, it is expected that CDRs of that MAb in such environments will nevertheless recognize the same epitope(s) as the MAb from which they are derived.

5 A "monoclonal antibody" refers to homogenous populations of immunoglobulins which are capable of specifically binding to WT1 protein. It is understood that WT1 protein may have one or more antigenic determinants, particularly in the amino acid sequence  
10 spanning amino acids 1-181 of SEQ ID NO: 4. The antibodies of the invention may be directed against one or more of these determinants. As used herein, a "cocktail" of these antibodies comprises any combination of the antibodies of the invention.

15 A "chimeric antibody" refers to a type of engineered or recombinantly produced antibody which contains naturally-occurring variable region light chain and heavy chains (both CDR and framework regions) derived from a non-human donor antibody, such as the MAbs described  
20 herein, in association with light and heavy chain constant regions derived from a human (or other heterologous animal) acceptor antibody.

A "humanized antibody" refers to a recombinantly produced antibody having its CDRs and/or other portions  
25 of its light and/or heavy variable domain framework regions derived from a non-human donor immunoglobulin, such as the MAbs of the present invention, the remaining immunoglobulin-derived parts of the molecule being derived from one or more human immunoglobulins. Such  
30 antibodies can also include a humanized heavy chain associated with a donor or acceptor unmodified light chain or a chimeric light chain, or vice versa.

A "bi-specific antibody" refers to an antibody derived from the Fab portions of two parent antibodies,  
35 each of which binds a separate antigen. The bi-specific

antibody is characterized by the ability to bind to two antigens, particularly, the antigens to which the parent antibodies bound.

5 A Fab fragment refers to a polypeptide containing one entire light chain and amino terminal portion of one heavy chain from an antibody, such as the MAbs of this invention. A F(ab')<sub>2</sub> fragment refers to the fragment formed by two Fab fragments bound by disulfide bonds.

10 II. Hybridoma Cell Lines and MAbs of the Invention

The hybridoma cell lines and monoclonal antibodies of the invention are produced by employing as antigen, a novel WT1-derived protein antigen, which contains only the N-terminal sequence of the WT1 protein. Desirably,  
15 the invention employs as an immunogen a WT1 containing protein antigen, referred to as WT1-6F [SEQ ID NO: 2], which contains amino acids 1-181 of the N-terminus of the native human WT1 sequence (see Fig. 1 and SEQ ID NO: 4). This antigen has been developed by the inventors and does  
20 not contain any of the zinc-finger region characteristic of the carboxyl terminal portion of the WT1 protein or any of the 17 amino acid insert of the splice variant of the protein (see Fig. 1). Additional details relating to the preparation and expression of the 6F antigen are  
25 provided in Example 1 below.

Generally, the hybridoma process involves generating a B-lymphocyte to the selected antigen, which B lymphocyte produces a desired antibody. Techniques for obtaining the appropriate lymphocytes from mammals  
30 injected with the target antigen, WT1-6F, are well known. Generally, the peripheral blood lymphocytes (PBLs) are used if cells of human origin are desired. If non-human sources are desired, spleen cells or lymph nodes from other mammalian sources are used. A host animal, e.g. a  
35 mouse, is injected with repeated doses of the purified

antigen, and the mammal is permitted to generate the desired antibody producing cells.

Thereafter the B-lymphocytes are harvested for fusion with the immortalizing cell line. Immortalizing cell lines are usually transformed mammalian cells, particularly cells of rodent, bovine and human origin. Most frequently, rat or mouse myeloma cells are employed. Techniques for fusion are also well known in the art and generally involve mixing the cells with a fusing agents, e.g. polyethylene glycol.

Immortalized hybridoma cell lines are selected by standard procedures, such as HAT selection. From among these hybridomas, those secreting the desired monoclonal antibody are selected by assaying the culture medium by standard immunoassays, such as Western blotting, ELISA, or RIA. Antibodies are recovered from the medium using standard purification techniques. See, generally, Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd edit., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). Alternatively, non-fusion techniques for generating an immortal antibody-producing hybridoma cell line may be employed to generate a hybridoma antibody, where possible, e.g. virally induced transformation.

The invention provides three exemplary hybridoma cell lines and the MAbs secreted therefrom produced using WT1-6F as the antigen. See Examples 2 and 3 below. These three hybridomas secrete antibodies termed H2, H7 and HC17, respectively. Each hybridoma was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A. ("ATCC") on March 31, 1994, pursuant to the provisions of the Budapest Treaty. The H2-secreting hybridoma was granted accession number ATCC 11598, the H7-secreting hybridoma was granted

accession number ATCC 11599, and the HC17-secreting hybridoma was granted accession number ATCC 11600.

The H2, H7 and HC17 antibodies are murine IgG<sub>1</sub> antibodies, and have been demonstrated to specifically  
5 bind WT1 protein and not to cross-react with the closely-related Egr1 family of proteins. All three monoclonal antibodies recognize the recombinant protein in ELISA assays, and full length WT1 protein in immuno-precipitation and Western blot analysis. Preliminary  
10 analysis suggests that at least two distinct epitopes in the WT1-6F protein are recognized by the three MABs. The MABs of this invention are characterized in more detail in Example 4 below.

A Western blot analysis was performed to test the  
15 ability of the three MABs to detect two recombinant proteins: 6F [SEQ ID NO: 2] which contains WT1 amino acids 1-181 of SEQ ID NO: 4, and WT91 which contains WT1 amino acids 85-173 of SEQ ID NO: 4. All three MABs detect the 6F recombinant protein [SEQ ID NO: 2]  
20 containing WT1 amino acids 1-181. However, only H2 and H7 detect the WT91 recombinant protein containing amino acids 85-173 of WT1, suggesting that H2 and H7 recognize an epitope within the WT1 amino acid sequence 85-173 and HC17 recognizes an epitope outside this region.

25 These MABs are useful as diagnostic reagents, and possibly as therapeutic reagents as described in more detail below.

### III. MAB Antibody Fragments

30 The present invention also includes functional fragments of the MABs defined above, preferably those derived from the H2, H7 and/or HC17 MABs of the invention. Such functional fragments include the heavy chain and light chain variable region polypeptides of the

MABs of the invention, and other fragments thereof, such as Fab fragments, F(ab)<sub>2</sub> fragments, Fv fragments and the like.

5        These fragments are useful as diagnostic reagents and as donors of sequences, including the variable regions and CDR sequences, useful in the formation of recombinant, chimeric, humanized or bi-specific antibodies. Techniques for generating such antibodies and antibody fragments are known in the art. For  
10    example, the functional fragments of the invention may be obtained using conventional genetic engineering techniques. See, generally, Sambrook et al., cited above. Alternatively, desired portions thereof, e.g. the CDR sequences, may be chemically synthesized.

15        These antibody functional fragments are useful in the assays of the invention to diagnose WT1 over-expression or inappropriate expression in specific tumors, which assays are described in more detail below. For example, by conjugating these antibody fragments to  
20    enzymes, such as horseradish peroxidase, these fragments may be employed in a conventional one-step detection assay.

#### IV.        Diagnostic Reagents and Kits

25        The invention includes kits of reagents for use in immunoassays, particularly sandwich immunoassays. Such kits include a solid phase support, a monoclonal antibody of the invention, a functional fragment thereof, or a cocktail thereof, and means for signal generation. The  
30    antibodies of the invention may be pre-attached to the solid support, or may be applied to the surface of the solid support when the kit is used. The signal generating means may come pre-associated with an antibody of the invention or may require combination with one or  
35    more components, e.g. buffers, antibody-enzyme

conjugates, enzyme substrates, or the like, prior to use. Kits may also include additional reagents, e.g. blocking reagents for reducing nonspecific binding to the solid phase surface, washing reagents, enzyme substrates, and  
5 the like. The solid phase surface may be in the form of microtiter plates, microspheres, or other materials suitable for immobilizing proteins. Preferably, an enzyme which catalyzes the formation of a chemiluminescent or colored product is a component of the  
10 signal generating means. Such enzymes are well known in the art.

Such kits are useful in the detection, monitoring and diagnosis of conditions characterized by over-expression or inappropriate expression of the WT1  
15 protein.

#### V. Diagnostic Assays

The MAbs, fragments, reagents and kits of the invention may be used with standard assay formats for the  
20 identification and diagnosis of conditions characterized by WT1 expression, over-expression or inappropriate expression, particularly in tumor/leukemic cells. The detection and measurement of WT1 expression in tissue that does not normally express WT1 or over-expression or  
25 inappropriate expression in tissue that does normally express WT1 may be accomplished by resort to several known techniques, e.g., immunofluorescence (detection of WT1 protein in fixed cells/tissues) and detection of WT1 protein of whole cell extracts by western analysis. Most  
30 particularly, the MAbs and other compositions of this invention may be used to detect WT1 expression in abnormal kidney and genitourinary development and cancers which over-express WT1, particularly, leukemias, mesothelioma, granulosa, prostate and ovarian cancers.

The reagents of the invention may also be used to monitor therapy of such conditions.

Desirably, the MABs and fragments thereof, when used as diagnostic reagents are conventionally labelled for use as molecular weight markers or for use in ELISAs, immunofluorescence, and other conventional assay formats for the measurement of WT1 in an appropriate biological sample. Suitable label systems are well known to those of skill in the art and include fluorescent compounds, radioactive compounds or elements, and a variety of enzyme systems. As used herein, suitable samples include, without limitation, whole blood, serum, plasma, tissue samples, bone marrow, and urine.

Advantageously, the MABs of the invention can be used to screen for the WT1 protein using standard antibody staining techniques, e.g. the avidin-biotin system, immunofluorescence, or the like. For example, a tissue, e.g. from a biopsy, is fixed on a slide using standard techniques. A selected MAB (or fragment thereof) of the invention is then applied to the slide and incubated under standard conditions, e.g. at room temperature for about 1 hour. A labelled anti-mouse antibody is then used for detection. Parallel experiments with positive and negative controls (minus MAB of invention) are performed.

Significantly, if the MABs of the invention avoid interference with MAB recognition by fixation of the tumor tissue with conventional reagents, e.g. paraformaldehyde and, preferably, methanol, these antibodies may be useful on routine pathology slides. For example, the ability of these monoclonal antibodies to detect prostate cancer cells has been demonstrated. Preliminary data has demonstrated that cocktails of these antibodies, e.g., H2/HC17 and H7/HC17, are particularly well suited for this purpose.



The MAbs, or functional fragments thereof, of the invention are useful in the detection of a condition characterized by over-expression of WT1 antigen, including leukemias, mesothelioma, and granulosa, or to  
5 differentiate such a condition from other conditions which exhibit similar clinical symptoms. For example, a Mab of the invention can differentiate a mesothelioma from other pleural tumors; such a use is clinically significant in view of the different prognoses for  
10 pleural tumors of non-adenocarcinoma origin and adenocarcinomas. Such a method involves obtaining a suitable biological sample from a patient, incubating the sample in the presence of a Mab or functional fragment thereof of the invention, and detecting the presence of  
15 binding of the Mab or fragment to the biological sample. The presence of binding above background levels detected in a non-WT1 expressing normal tissue sample indicates the presence of a mesothelioma. Any tissue or established cell line which does not express WT1 mRNA may  
20 serve as a standard for negative expression of WT1 protein, including those described above in the background.

Alternatively, the MAbs and fragments thereof of the invention are useful to monitor a course of treatment for  
25 a condition characterized by over-expression or inappropriate expression of the WT1 antigen. For example, active leukemia (e.g. in blast crisis) cells express WT1, while inactive leukemic cells do not express WT1. Thus, during or following a treatment cycle, a  
30 biological sample from the leukemia patient is periodically tested in an assay of the invention to monitor residual leukemic disease. The lack of, or reduction of levels of, binding of a Mab or fragment of the invention to the sample indicates that the course of  
35 treatment, e.g., chemotherapy, is successful in reducing

the tumor or cancer. Similarly, the MAbs and fragments of the invention may be used to detect leukemic blast cells in purged or unpurged hematopoietic stem cell preparations intended for use in bone marrow  
5 transplantation.

It is anticipated that one of skill in the art of diagnostic assays may devise other series of steps utilizing the MAbs or fragments of this invention to accomplish the detection of levels of WT1 expression  
10 indicative of disease. Such assay formats are known within the art, and are simply a matter of selection. This invention is not limited by the particular assay format or assay steps employed in the diagnosis of inappropriate expression of WT1 protein in biological  
15 samples.

Because the MAbs H2, H7, and HC17 were raised to a region of the WT1 amino acid sequence that is unique to the amino terminal portion of WT1 and does not contain the zinc finger DNA binding domains, these MAbs and  
20 fragments have little potential for crossreactivity with non-WT1 proteins, unlike known other WT1 polyclonal and monoclonal MAbs. For example, these MAbs do not cross-react with the Egr family of proteins. Thus they permit an unambiguous positive detection of WT1 expression in  
25 biological samples.

The advantages of using these MAbs for such diagnosis in comparison to the use of the known monoclonal and polyclonal antibodies of the art rely in the defined specificity of the MAbs for the amino  
30 terminal sequence of WT1, their uniform binding affinity and their lack of cross-reactivity as described above.

V. Therapeutic Use of Mabs of this Invention

Further, if these Mabs of the invention are have the ability to internalize into the nucleus of the cell which expresses WT1 [see, e.g., United States Patent No. 5,296,348, issued March 22, 1994, incorporated by reference herein], they may also be employed in the treatment of such WT-1 expressing tumors or cancers. For example, these Mabs, other antibody types such as chimeric or humanized antibodies, or fragments which share the binding affinity or specificity of the whole Mab may be used to deliver toxins or therapeutic agents to the tumor or metastasis sites.

These Mabs, other antibodies and fragments of the present invention may also be employed in other therapeutic methods known to those of skill in the art.

The following examples illustrate the characterization and uses of the antibodies of the invention. These examples are illustrative only and do not limit the scope of the invention.

20

Example 1 - Preparation of the WT1-6F Antigen

A. Cloning Strategy

A recombinant protein containing the first 181 amino acids of the human WT1 was produced to use as an antigen in the preparation of WT1 specific antibodies as follows.

The amino terminus of WT1 was subcloned from 7Zf+WT1, a synthetic full-length human WT1 nucleotide sequence described in Morris et al, cited above. Briefly, the nucleotide sequence encoding the full-length protein was constructed from the partial human WT1 cDNA clone WT33 [Call et al, Cell, 60:509-520 (1990)]. The WT1 amino acids 1-84 plus an overlapping 20 amino acid segment were synthesized using overlap-extension

polymerase chain reaction. The nucleotides specifying amino acid codons were optimized for expression in *E. coli*.

The resulting synthetic DNA fragment (320 bp) was fused to the 5' end of WT33 at a unique Bst XI site centered at position WT1 amino acid 101 of SEQ ID NO: 4 (nucleotide 50 of WT33). A Cla I-Eco RI fragment was subcloned into pGem7Zf+ (Promega, Madison, WI) to produce 7Zf+WT1. From this plasmid, a Nco I-Bam HI fragment was isolated and subcloned into a modified pet11d vector (Novagen, Madison, WI).

The pet11d vector was modified by digesting with Nco I and ligating to synthetic, double-stranded oligonucleotides which contained the following 5' overhangs complementary to a Nco I restriction site to produce 6XHISpet11d:

5'-CATGAGAGGATCGCATCACCATCACCATCACTC 3'[SEQ ID NO: 5]  
3' TCTCCTAGCGTAGTGGTAGTGGTAGTGAGGTAC-5'[SEQ ID NO: 6].

The synthetic oligonucleotide introduces nucleotide codons that encode the amino acids MRSHHHHHH of SEQ ID NO: 2 to produce an in-frame 5' hexa-histidine fusion protein that maintains a single Nco I restriction site at the 3' end of the sequence. The 5' hexa-histidine encoding region facilitates protein purification [Abate et al, Proc. Natl. Acad. Sci. USA, 87:1032 (1990)].

The Nco I-Bam HI fragment of 7Zf+WT1 containing the amino terminus of WT1 was subcloned into 6XHISpet11d digested with Nco-I and Bam HI to create pet11d-6F.

#### B. Expression in *E. coli* and Purification

The bacterial strain BL21 (Novagen, Madison, WI) was transformed with the pet11d-6F DNA. Protein expression was induced in a logarithmically growing bacterial culture with 1 mM isopropyl- $\beta$ -thiogalactopyranoside (IPTG) for two to three hours at 37°C.

Bacteria were harvested by centrifugation, lysed in 6 M guanidine-HCl, 50 mM sodium phosphate, pH 8.0 for 2 hours at room temperature or overnight at 4°C, and clarified by centrifugation at 40,000 x g for 30 minutes.

5 The histidine fusion recombinant protein WT1-6F was purified by a one step affinity binding of the hexahistidines to the nickel-chelate affinity resin NTA-agarose (Qiagen, Chatsworth, CA) using recommended procedures. The purified protein was renatured by  
10 dialysis into phosphate buffered saline with 5% glycerol.

Purity of the protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows. The purified protein was renatured by dialysis into PBS containing 5% glycerol and analyzed on  
15 a 10% SDS polyacrylamide gel. Proteins were stained with Coomassie blue. The 6F recombinant protein was shown to be homogenous, migrating under denaturing conditions as a 28 kDa protein.

#### C. The WT1-6F Antigen

20 The 6F amino acid sequence is encoded by a synthetic nucleotide sequence shown in Fig. 2 [SEQ ID NOS: 2 and 1]. The 6F nucleotide sequence was derived from the synthetic full-length human WT1 sequence [Morris et al, cited above and SEQ ID NOS: 3 and 4]. As  
25 illustrated in Fig. 2, the recombinant 6F antigen contains amino acids 1-181 of the human WT1 sequence [SEQ ID NO:4] as well as amino acids at both the amino and carboxyl ends, which sequences were introduced during cloning. The entire 6F amino acid sequence is shown in  
30 Fig. 2 [SEQ ID NO: 2]. Amino acids 1-11 (MRGSHHHHHHS) of SEQ ID NO: 2 were added to produce a histidine fusion protein to facilitate purification of the recombinant protein. Amino acids 193-210 of SEQ ID NO: 2 are not WT1 sequences, but were added from the vector during cloning.  
35 Note that nucleotides 1-333 [SEQ ID NO: 1] are synthetic

sequences optimized for protein translation in *E. coli*; the remaining nucleotides are derived from the human WT33 cDNA clone. This does not change the human WT1 amino acid sequence, but increases efficiency of protein expression in *E. coli* [Rauscher et al, Science, 250:1259-1262 (1990), Abate et al, Proc. Natl. Acad. Sci., 87:1032-1036 (Feb. 1990)].

A second recombinant protein, WT91 (described in Morris et al, cited above) contains the amino acids 85-173 of SEQ ID NO: 4.

#### Example 2 - Preparation of Antisera and Immunization

Rabbit polyclonal antisera was produced by CoCalico Biologicals, Inc. Rabbits were immunized subcutaneously with 100  $\mu$ g of 6F recombinant protein of Example 1 and boosted at two to three week intervals. The rabbit sera was used without further purification.

#### Example 3 - Preparation of Monoclonal Antibodies

Fifty micrograms of purified recombinant protein of Example 1 was injected subcutaneously into the hind footpads of BALB/c mice every two weeks for a total of three injections. Sera was collected from the tail, and tested for WT1 specific antibodies by immunoprecipitation of <sup>35</sup>S-methionine labeled *in vitro* translated human WT1 protein.

Two weeks later, 50  $\mu$ g of protein in 200  $\mu$ l of saline was injected intravenously followed by removal of each animal's spleen. Spleen cells were fused with a myeloma cell line, P3X63AG8/653 [ATCC CRL 1580], using standard techniques.

The resulting hybridomas producing MAbs H2, H7 and HC17 were screened in a two step process. Positive clones were initially identified using an enzyme-linked immunosorbent assay (ELISA) against the 6F recombinant

protein. Secondary screening was carried out using immunoprecipitation of full length WT1 protein produced by *in vitro* translation (IVT). These experiments demonstrated that the MAbs H2, H7 and HC17 specifically recognize the WT1 protein and that they appear to recognize distinct epitopes on the WT1 protein.

1. Immunoprecipitation

Full length WT1 was expressed *in vitro* from by transcribing RNA from Eco RI linearized vector 7Zf+WT1 with SP6 RNA polymerase, and translating protein in rabbit reticulocyte lysate with <sup>35</sup>S-methionine. The <sup>35</sup>S-methionine labeled protein is 55 kDa and is specifically immunoprecipitated by rabbit polyclonal anti-6F sera, and by the mouse monoclonal antibodies H2, H7, and HC17.

Immunoprecipitations were done as previously described in Morris et al, cited above. Briefly, IVT WT1 was added to radioimmunoprecipitation buffer with protease inhibitors (RIPA: 10 mM Tris-Cl pH 7.4, 150 mM sodium chloride, 1 mM ethylenediamine-tetraacetic acid (EDTA), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.1 mM phenylmethylsulfonic acid (PMSF), 2 µg/ml leupeptin and 2 µg/ml aprotinin) along with antibodies and incubated 90 minutes at 4°C. Either 30 µl of 10% *Staphylococcus A* (Pansorbin, Calbiochem, San Diego, CA) or 100 µl of 50% Protein A Sepharose (Pharmacia, Piscataway, NJ) was added and incubated for 15 minutes (Staph A) or 30 minutes (Protein A). The immune complexes were collected by centrifugation in the microfuge and washed with 0.5-1.0 ml of RIPA 3 to 4 times. The immunoprecipitated proteins were analyzed on 10 or 15% SDS-polyacrylamide gels and visualized by autoradiography.

The resulting SDS PAGE gel demonstrated that MAbs of this invention immunoprecipitate WT1 expressed by *in vitro* transcription and translation.

## 2. Baculovirus expression of full length WT1

The full length WT1 protein encoding sequence was subcloned from 7Zf+WT1 into a baculovirus expression vector. Sf9 insect cells were infected with WT1-  
5 baculovirus and cells harvested 48-96 hours following infection. Cells were pelleted by centrifugation, washed three times in PBS. Whole cell lysates were prepared by  
10 lysing a cell pellet in 10 times the cell pellet volume with Laemmli loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol).

Ten  $\mu$ l of WT1 lysate were analyzed on a 10% SDS-polyacrylamide gel. Western analysis of protein was performed as follows. A whole cell lysate of Sf9 cells  
15 expressing baculovirus encoded WT1 protein was separated on a 10% SDS-polyacrylamide gel and transferred to 0.45  $\mu$ m BA 85 nitrocellulose (Schleicher and Schuell, Keene, NH) using semi-dry electroblot transfer for 60-90 minutes at 4 mAmps/cm<sup>2</sup>. Molecular weight standards were cut from  
20 the blot and stained with Amido black and the nitrocellulose filter blot was blocked in 5% BSA-PBS for 60 minutes at room temperature or overnight at 4°C. The primary antibody was diluted in blocking buffer (rabbit anti-6F 1:400; the monoclonal antibodies of the invention  
25 1:500 or 1:1000) and added to filters for 30 to 60 minutes at room temperature.

Filters were rinsed briefly twice in wash buffer (PBS, 0.1% BSA, 1% Tween 20) and three times for  
30 10 minutes each while shaking vigorously. Soluble protein A conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) was diluted 1:5000 in 5% BSA-PBS and incubated for 30 minutes at room temperature. Filters were washed as before, rinsed in PBS, and  
35 incubated with a 1:1 mixture of the ECL substrates A and B (Amersham, Arlington Heights, IL) for 1 minute at room



temperature. Filters were removed from the liquid, excess moisture drained, and wrapped in Saran wrap and immediately exposed to film (average exposure 15 seconds to 3 minutes).

5                   The gels revealed that the polyclonal and monoclonal antibodies of this invention specifically detect a 55 kDa protein in Sf9 cells transfected with WT1 baculovirus expression vector and not cells mock transfected.

10

Example 4 - Characterization of Murine MAbs H2, H7 and HC17

To determine whether the WT1 monoclonal antibodies of the present invention detect different epitopes within the first 181 amino acid of the 6F antigen, purified recombinant proteins 6F (WT1 amino acid 1-181) and WT91 (WT1 amino acid 85-173) were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. Western blot analysis was performed as described in Example 3.

20

Polyclonal antibodies were diluted 1:400 and monoclonal antibodies diluted 1:500. The polyclonal antisera recognizes both the 6F and WT91 recombinant proteins. The monoclonal antibodies H2 and H7 recognize both 6F and WT91 recombinant proteins, suggesting they detect an epitope with amino acid 85-173 of WT1 [SEQ ID NO:4]. HC17 does not detect the WT91 recombinant protein indicating that it recognizes an epitope outside of this region.

30

35

Example 5 - Detection of WT1 Protein in Human Acute Leukemias

The following study demonstrates that a MAb of the invention, H2, is able to distinguish between leukemic blast cells and normal mononuclear cells by detecting the WT1 protein in nuclei of leukemic blast cells. No WT1 protein was detected in the nuclei of normal mononuclear cells or mononuclear cells by either immunofluorescence or by conventional reverse-transcriptase polymerase chain reaction (RT-PCR) techniques.

A. Samples

Mononuclear cell preparations of 110 adult leukemia patients were examined in this study, T-cell acute lymphoblastic leukemias (T-ALL) had been diagnosed in 27, common acute lymphoblastic leukemias (c-ALL) in 28, pre-pre-B cell acute lymphoblastic leukemias (ppB-ALL) in 8, acute myelogenous leukemias (AML) in 40, chronic myelogenous leukemias in blast crisis (one lymphatic and three myeloid CML-BC) in 4 and chronic myelogenous leukemias in chronic phase (CML-CP) in 3 patients. Controls were 4 patients with reactive bone marrow aspirates who had fever of unknown origin (H.M., G.S.), anemia secondary to iron deficiency (V.H.) or limited-disease esophageal cancer with no morphological evidence of bone marrow infiltration (H.F.).

Mononuclear cells were isolated from bone marrow aspirates or in a few cases from peripheral blood samples by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Freiburg, Germany). Also, peripheral mononuclear cells enriched with CD34<sup>+</sup> hematopoietic progenitors were obtained from five patients (S.K., S.Kt., K.D., N.G., H.G.) who had solid cancer with no morphological evidence of bone marrow infiltration. Their mononuclear cells had been harvested by leukapheresis during the recovery phase following a

course of progenitor-cell-mobilizing chemotherapy and G-CSF. Furthermore, a 91% pure peripheral CD34<sup>+</sup> hematopoietic progenitor cell suspension was prepared from the leukapheresis product of a patient (G.M.) suffering from plasmacytoma.

The number of peripheral CD34<sup>+</sup> progenitors was determined using a FACScan cytofluorometer. At least 10<sup>5</sup> CD34<sup>+</sup> vital cells per sample were available for testing. In addition, nucleated blood cells of twenty patients with non-neoplastic disease were isolated using a red blood cell lysis-buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, and 0.1 mM EDTA). The leukemia cell line K562 [ATCC CCL 243] served as the positive control in detection of wt1 mRNA and in immunofluorescence studies.

#### B. Indirect Immunofluorescence Assay

For the indirect immunofluorescence assay, mononuclear cells of bone marrow were isolated as already described. In addition, a 91% pure CD34<sup>+</sup> hematopoietic progenitor cell suspension was prepared from the leukapheresis product of a patient (G.M.) suffering from plasmacytoma. Prior to leukapheresis, she underwent peripheral stem-cell mobilization with chemotherapy (VAD-protocol) and G-CSF.

An aliquot taken from the leukapheresis product contained 2.5 x 10<sup>8</sup> mononuclear cells and, according to FACS analysis [M. Notter et al, Blood, 82:3113 (1993)], 8.75 x 10<sup>6</sup> CD34<sup>+</sup> hematopoietic progenitor cells. First, T-lymphocytes and myeloid cells were depleted using paramagnetic microbeads coupled with mouse anti-human CD3 and CD33 MAbs (Miltenyi, Cologne, Germany). Using a B2 column (Miltenyi), the cells were sorted according to the manufacturer's instructions. Subsequently, CD34<sup>+</sup> hematopoietic progenitor cells were isolated using the CD34 Progenitor Isolation Kit (QBEND/10) from Miltenyi. After removal of unbound MAb by washing, cells were

passed twice over a Mini MACS column (without flow resistor, Miltenyi). The 8G12-PE MAb (Becton Dickinson, Heidelberg, Germany) was used to determine the purity of the final CD34<sup>+</sup> cell suspension, which was 91% with a yield of 39%.

One fraction of the cell preparations was processed according to the RT-PCR protocol described to detect the wt1 transcript. Another fraction was used in the immunofluorescence assay. K562 cells served as positive controls. For detection of the nuclear protein WT1, 5 x 10<sup>4</sup> mononuclear cells were cytocentrifuged onto glass slides and air-dried for 2 hours. To destroy cellular membranes, the cells were exposed to pure methanol for 30 minutes at 4°C and then washed twice in PBS. The cells were incubated for 45 minutes at 4°C with the mouse antihuman WT1 MAb H2, produced as described in Example 3 above, or a negative control MAb (MAb 425) recognizing the EGF-receptor [Rodeck et al, Cancer Res., 47:3692 (1987)]. The cells were washed again in PBS and incubated for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated goat antimouse F(ab')<sub>2</sub> fragments (Immunotech, Marseille, France). After washing in PBS, cells were embedded in PBS-glycerin and analyzed by fluorescence microscopy (Axiophot, 1000x, Zeiss, Oberkochen, Germany). Results are reported below in Table 1.

Table 1

|    | <u>Diagnosis</u>                    | <u>Patient Initials</u> | <u>wt1 mRNA Expression</u> | <u>Nuclear Immunofluorescence</u> |                |
|----|-------------------------------------|-------------------------|----------------------------|-----------------------------------|----------------|
|    |                                     |                         |                            | <u>MAb H2</u>                     | <u>MAB 425</u> |
| 5  | <u>ALL</u>                          |                         |                            |                                   |                |
|    | pre-pre-B-ALL                       | C.R.                    | yes                        | #yes                              | \$no           |
|    | c-ALL                               | R.P.                    | yes                        | yes                               | no             |
|    | c-ALL                               | F.G.                    | yes                        | yes                               | no             |
|    | c-ALL                               | W.T.                    | no                         | no                                | no             |
| 10 | T-ALL                               | A.D.                    | yes                        | no                                | no             |
|    | T-ALL                               | M.S.                    | no                         | no                                | no             |
|    | <u>AML</u>                          |                         |                            |                                   |                |
|    | AML-FAB-M2                          | M.E.                    | yes                        | yes                               | no             |
| 15 | AML-FAB-M4                          | A.M.                    | yes                        | yes                               | no             |
|    | AML-FAB-M2                          | H.K.                    | yes                        | no                                | no             |
|    | AML-FAB-M1                          | H.L.                    | no                         | no                                | no             |
|    | <u>Controls</u>                     |                         |                            |                                   |                |
| 20 | K562 cells                          |                         | yes                        | yes                               | no             |
|    | CD34+91% pure progenitor cells G.M. |                         | no                         | no                                | no             |
| 25 | normal blood mononuclear cells      |                         | no                         | no                                | no             |

# indicates more than 30% of cells show a strong nuclear fluorescence.

30 \$ indicates no cells show nuclear fluorescence.

The indirect immunofluorescence assay with the MAb H2 directed against the WT1 nuclear protein disclosed a strong and specific nuclear fluorescence in blast cells from 3 of 6 ALL patients and 2 of 4 AML patients tested (Table 1). No nuclear immunofluorescence was observed in 3 ALL patients, one with (A.D.) and two without wt1 gene expression. In mononuclear cell preparations from 4 AML patients a nuclear immunofluorescence with MAb H2 was found in 2 cases and both tested positive for wt1 mRNA expression using RT-PCR. While blast cells of one AML patient did not express the wt1 mRNA and had no nuclear immunofluorescence with MAb H2, those of another AML patient did show transcription of the wt1 mRNA but no nuclear immunofluorescence (H.K., Table 1). K562 cells

showed strong nuclear immunofluorescence with MAb 6F-H2, whereas normal mononuclear blood cells and cells of a 91% pure CD34<sup>+</sup> hematopoietic progenitor cell suspension did not (Table 1). There was no nuclear immunofluorescence detectable using the negative control MAb 425 (Table 1). In normal blood granulocytes, cytoplasmic but no nuclear fluorescence was found with MAb H2 and regarded as unspecific (data not shown).

Immunofluorescence using MAb H2 confirms RT-PCR data, and shows detection of the WT1 protein in nuclei of leukemic blast cells but not in those of normal mononuclear cells or mononuclear cells enriched with CD34<sup>+</sup> hematopoietic progenitors.

Expression of protein occurs following the transcription of mRNA message from the double stranded DNA. This mRNA is translated into a protein. Detectable mRNA indicates that the necessary "intermediate" is present and potentially capable of being translated into protein. However, this correlation does not always occur and the presence of mRNA does not necessarily mean the protein is being produced. Therefore, immunofluorescence detects protein expression and is the preferable assay system.

#### Example 6 - Detection of WT1 Protein in Malignant Mesotheliomas

##### A. Cell Lines

The mesothelioma cell lines (ML1-ML19) used in the study were all developed from human mesothelioma tumors diagnosed using conventional immunohistochemical tests. Cell lines ML-10 and ML-16 were established by explant culture at the University of Pennsylvania [W. R. Smythe et al, Ann. Thorac. Surg., 57(6):1395-1401 (1994)]. Both cell lines have been passaged over 25 times without evidence of senescence, grow as tumors in

immunodeficient mice, and show a staining pattern characteristic of mesothelioma with lack of staining with LeuM1 and carcinoembryonic antigen (CEA) antibodies. Cell lines ML1-ML8 were developed in the Surgical  
5 Oncology Laboratory at the National Cancer Institute (USA). Mesothelioma cell lines, (ML11-ML15) and lung cancer lines (LL5-LL8) were provided by Dr. Carmen Allegra from the Medical Oncology Branch, NCI-Navy, National Naval Medical Center. Cell lines: ML9 (H-Meso),  
10 ML17, ML18 and ML19 were provided by Dr. Joseph Testa from Fox Chase Cancer Institute, Philadelphia, PA. Normal mesothelial cells were developed from explants derived from non-malignant visceral pleural tissue obtained at surgery.

15           These cell lines were maintained in RPMI-40 media (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal calf bovine serum, non-essential amino acids (10 mM), L-Glutamine (200 mM), penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml). The six lung cancer cell  
20 lines, LL1 (A549), LL2 (Calu-1), LL3 (Calu-3), LL4 (Calu-6), LL9 (SK-LU-1), LL10 (SK-MES-1), were purchased from American Type Culture Collection (ATCC) and cultured per instructions. Normal bronchial epithelial cells [S. A. Mette et al, Am. J. Respir. Cell. Mol. Biol., 8:562-572  
25 (1993)] (HBE4) and human umbilical vein endothelial cells were cultured as described in S. M. Albelda et al, J. Clin. Invest., 83:1992-2002 (1989)].

#### B. Transfection Protocol

To generate a positive control for cellular  
30 localization studies of WT1 protein, COS-1 cells (ATCC) were either seeded at  $5 \times 10^4$  cells/cm<sup>2</sup> onto 1% gelatin-coated coverslips or at  $5 \times 10^5$  cells in a 35 mm dish and maintained in DMEM (Gibco-BRL, Gaithersburg, MD) plus 10% fetal bovine serum. Twenty-four hours later, 2.5  $\mu$ g of  
35 pCMVhuWT1cDNA, an expression vector described previously

[Morris et al, cited above] was transfected into the cells by the calcium phosphate-mediated co-precipitation method [J. Sambrook et al, cited above]. Three days later the cells on the coverslips were processed for immunofluorescence staining with WT1 antibody and cells in 35 mm dish were harvested for immunoblot analysis which is described below.

C. Human Tissue and Tumor Specimens

Excess tissue specimens from normal organs, 9 mesothelioma tumors (Table 2), and 9 non-small cell lung carcinomas (NSCLC) were obtained freshly at the time of surgery and either immediately frozen in liquid nitrogen or frozen on dry ice after embedding in O.C.T. compound (Miles Scientific, Elkhart, IN). Samples were stored at -70°C until further analysis. All diagnoses for the tumors were made by the pathologists at the University of Pennsylvania based on conventional histological and clinical criteria. Mesothelioma tumors were stained immunohistochemically and were characteristically negative for LeuM1 and CEA. Results are reported in Table 2 below.

Table 2

| <u>Sample</u> | <u>Age</u> | <u>Sex</u> | <u>Histologic Type</u>                 |
|---------------|------------|------------|--|
| MT1           | 56         | M          | Epithelial malignant mesothelioma (MM) |
| 25 MT2        | 69         | F          | Epithelial MM                          |
| MT3           | 59         | F          | Mixed MM                               |
| MT4           | 51         | M          | Spindle Cell MM                        |
| MT5           | 61         | M          | Mixed MM                               |
| MT6           | 72         | M          | Fibrosarcomatous MM                    |
| 30 MT7        | 70         | M          | Inflammatory MM                        |
| MT8           | 65         | M          | Epithelial MM                          |
| MT9           | -          | 0          | Benign fibrous tumor                   |



#### D. Immunoblot Analysis

To determine if the WT1 protein was expressed in mesothelioma cell lines, immunoblotting experiments were performed, as follows, on nuclear extracts using the H2 anti-WT1 MAb prepared as described in Example 3 above.

Nuclear extracts were prepared from cell lines using standard techniques [F. M. Ausubel et al, In Current Protocols in Molecular Biology, John Wiley and Sons, New York (1991)]. The nuclear pellet was collected by centrifugation at 4000 rpm for 15 minutes at 4°C, resuspended in 5 times the pellet volume in electrophoresis sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, pH 6.8), and boiled for 5 minutes. Seventy-five  $\mu$ l of nuclear extract was applied on a 10% SDS-polyacrylamide gel under reducing conditions. The separated proteins were transferred to a nitrocellulose membrane which was developed as previously described [K. A. Knudsen et al, Exp. Cell. Res., 157:218-226 (1985)] using anti-WT1 as a primary antibody and an alkaline phosphatase-coupled anti-mouse as the secondary antibody.

The H2 MAb recognized a 52 KDa protein from the COS-1 cells transfected with pCMVhuWT1cDNA. No WT1 expression was seen in non-transfected cells or in LL1, a lung cancer cell. However, in the ML17, ML13, ML16, and ML14 mesothelioma cell lines the antibody recognized two (52 and 55 KDa) proteins in varying amounts.

#### E. Immunolocalization Studies

##### 1. Immunofluorescence

In order to determine the cellular location of the WT1 protein and to confirm the immunoblotting experiments, immunofluorescence staining was performed on some of the mesothelioma cell lines, as follows.

Cell lines ML13 and ML16 which express elevated levels of WT1 mRNA (determined using conventional RT-PCR techniques) were analyzed and LL1 used as a negative control, since it expressed almost no WT1 mRNA. An isotyped matched monoclonal antibody against the endothelial cell specific molecule, PECAM-1 was used as a non-reactive control. Confluent monolayers of cells grown on glass coverslips coated with 1% gelatin were processed as previously described [S. M. Albelda et al, cited above]. Immunofluorescence studies were performed with a 1:250 dilution of anti-WT1 ascites and a 1/200 fluorescein-conjugated anti-mouse antibody (Cappel Labs, Malvern, PA). The coverslips were evaluated under epifluorescence. COS-1 cells grown on coverslips and transfected with pCMVhuWT1cDNA, were used as a positive control.

COS-1 cells transfected with pCMVhuWT1cDNA stained strongly with the monoclonal anti-WT1 H2 with expression confined to the nucleus. In contrast, the untransfected COS-1 cells showed only baseline fluorescence. A similar nuclear staining pattern has been seen in COS-1 cells transfected with WT1cDNA and stained with a polyclonal anti-WT1 antibody [J. F. Tet al, cited above]. Clear nuclear staining with the anti-WT1 H2 MAb was also seen in the ML13 and the ML16 mesothelioma cell lines. In contrast, the lung cancer cell line (LL1) which did not express any WT1 mRNA did not stain positively with the anti-WT1 antibody. No appreciable staining was seen with the control antibody on any of the cell lines tested indicating the specificity of WT1 MAb.

## 2. Immunohistochemistry

In addition to evaluating WT1 protein expression in cell lines, the WT1 protein expression was evaluated in tissues by immunohistochemical staining.

Frozen sections from 5 mesotheliomas and 5 NSCLC solid tumor specimens were stained with anti-WT1 MAb and a control MAb.

For immunohistochemistry, thin sections (5  
5  $\mu\text{m}$ ) were prepared from frozen tissues embedded in O.C.T., fixed in acetone at  $-20^{\circ}\text{C}$  for 5 minutes and stored at  $-70^{\circ}\text{C}$ . Prior to staining, the sections were blocked with 5% horse serum in PBS and washed twice in PBS at room temperature. The sections were incubated with a 1/1000  
10 dilution of primary WT1 monoclonal antibody diluted in PBS/4% bovine serum albumin (BSA) for 1 hour at room temperature. Sections were washed twice in PBS/4% BSA, and then incubated for 30 minutes with a 1/1000 diluted biotinylated IgG horse antibody to mouse. The  
15 streptavidin-biotin ABC peroxidase detection system (Vector, Burlingame, CA) was applied, followed by a 2 minute incubation with 3-amino-9-ethylcarbazole (AEC) (Zymed, San Francisco, CA) as the substrate. The sections were mounted and evaluated microscopically.

20 Strong, primarily nuclear, staining was noted in a subset of identifiable neoplastic cells (5-10%) in all 5 mesothelioma tumors. Nuclear staining was not observed with a control MAb. Immunohistochemical staining of WT-1 was not observed, in any of the 5 non-  
25 small cell lung carcinomas examined as illustrated for LC4 and LC8.

#### F. Results

Immunohistochemical staining of both the mesothelioma tumors and the cell lines with the anti-WT1  
30 monoclonal antibody, H2, further revealed that WT1 protein is expressed abundantly. As predicted for a transcription factor, the WT1 protein localized to the nucleus in a proportion of cells in culture and in tumors. Although the staining of WT1 protein has been  
35 observed in F9 embryonic carcinoma cells and in K562

cells [A. Telerman et al, Oncogene, 8:2545-2548 (1992)], immunohistochemical localization of WT1 in human tissues has not been previously reported. The general pattern of the expression WT1 protein was somewhat heterogeneous in mesothelioma tumors, however, WT1 was consistently  
5 expressed in at least some cells of all the tumors examined. Immunoblot analysis of nuclear extracts from mesothelioma cell lines revealed the presence of a 52 KDa and a 54 KDa sized WT1 proteins. Whether the two  
10 proteins represent alternatively spliced WT1 iso-forms [D. A. Haber et al, Proc. Natl. Acad. Sci. USA, 88:9618-9622 (1991)] or a single form differently processed in the cancer cells is not known.

Numerous modifications and variations of the present  
15 invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims  
20 appended hereto.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: The Wistar Institute of Anatomy and  
Biology
- (ii) TITLE OF INVENTION: WT1 Monoclonal Antibodies  
and Methods of Use Therefor
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
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  - (D) STATE: Pennsylvania
  - (E) COUNTRY: USA
  - (F) ZIP: 19477
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0,  
Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: WO
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/234,783
  - (B) FILING DATE: 28-APR-1994
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Bak, Mary E.
  - (B) REGISTRATION NUMBER: 31,215
  - (C) REFERENCE/DOCKET NUMBER: WST48PCT
- (ix) TELECOMMUNICATION INFORMATION:
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  - (B) TELEFAX: 215-540-5818

36

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 633 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..630

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ATG | AGA | GGA | TCG | CAT | CAC | CAT | CAC | CAT | CAC | TCC | ATG | GGT | 39  |
| Met | Arg | Gly | Ser | His | His | His | His | His | His | Ser | Met | Gly |     |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     |
| TCC | GAC | GTT | CGT | GAC | CTG | AAC | GCA | CTG | CTG | CCG | GCA | GTT | 78  |
| Ser | Asp | Val | Arg | Asp | Leu | Asn | Ala | Leu | Leu | Pro | Ala | Val |     |
|     | 15  |     |     |     | 20  |     |     |     |     |     | 25  |     |     |
| CCG | TCC | CTG | GGT | GGT | GGT | GGT | GGT | TGC | GCA | CTG | CCG | GTT | 117 |
| Pro | Ser | Leu | Gly | Gly | Gly | Gly | Gly | Cys | Ala | Leu | Pro | Val |     |
|     |     |     | 30  |     |     |     |     | 35  |     |     |     |     |     |
| AGC | GGT | GCA | GCA | CAG | TGG | GCT | CCG | GTT | CTG | GAC | TTC | GCA | 156 |
| Ser | Gly | Ala | Ala | Gln | Trp | Ala | Pro | Val | Leu | Asp | Phe | Ala |     |
| 40  |     |     |     | 45  |     |     |     |     |     | 50  |     |     |     |
| CCG | CCG | GGT | GCA | TCC | GCA | TAC | GGT | TCC | CTG | GGT | GGT | CCG | 196 |
| Pro | Pro | Gly | Ala | Ser | Ala | Tyr | Gly | Ser | Leu | Gly | Gly | Pro |     |
|     |     | 55  |     |     |     |     | 60  |     |     |     |     | 65  |     |
| GCA | CCG | CCG | CCG | GCA | CCG | CCG | CCG | CCG | CCG | CCG | CCG | CCG | 234 |
| Ala | Pro | Pro | Pro | Ala | Pro | Pro | Pro | Pro | Pro | Pro | Pro | Pro |     |
|     |     |     |     | 70  |     |     |     |     |     | 75  |     |     |     |
| CCG | CAC | TCC | TTC | ATC | AAA | CAG | GAA | CCG | AGC | TGG | GGT | GGT | 273 |
| Pro | His | Ser | Phe | Ile | Lys | Gln | Glu | Pro | Ser | Trp | Gly | Gly |     |
|     | 80  |     |     |     |     | 85  |     |     |     |     | 90  |     |     |
| GCA | GAA | CCG | CAC | GAA | GAA | CAG | TGC | CTG | AGC | GCA | TTC | ACC | 312 |
| Ala | Glu | Pro | His | Glu | Glu | Gln | Cys | Leu | Ser | Ala | Phe | Thr |     |
|     |     |     | 95  |     |     |     |     | 100 |     |     |     |     |     |
| GTT | CAC | TTC | TCC | GGC | CAG | TTC | ACT | GGC | ACA | GCC | GGA | GCC | 351 |
| Val | His | Phe | Ser | Gly | Gln | Phe | Thr | Gly | Thr | Ala | Gly | Ala |     |
| 105 |     |     |     |     | 110 |     |     |     |     |     | 115 |     |     |

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|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| TGT | CGC | TAC | GGG | CCC | TTC | GGT | CCT | CCT | CCG | CCC | AGC | CAG | 390 |
| Cys | Arg | Tyr | Gly | Pro | Phe | Gly | Pro | Pro | Pro | Pro | Ser | Gln |     |
|     |     | 120 |     |     |     |     | 125 |     |     |     |     | 130 |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| GCG | TCA | TCC | GGC | CAG | GCC | AGG | ATG | TTT | CCT | AAC | GCG | CCC | 429 |
| Ala | Ser | Ser | Gly | Gln | Ala | Arg | Met | Phe | Pro | Asn | Ala | Pro |     |
|     |     |     | 135 |     |     |     |     |     | 140 |     |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TAC | CTG | CCC | AGC | TGC | CTC | GAG | AGC | CAG | CCC | GCT | ATT | CGC | 468 |
| Tyr | Leu | Pro | Ser | Cys | Leu | Glu | Ser | Gln | Pro | Ala | Ile | Arg |     |
|     | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AAT | CAG | GGT | TAC | AGC | ACG | GTC | ACC | TTC | GAC | GGG | ACG | CCC | 507 |
| Asn | Gln | Gly | Tyr | Ser | Thr | Val | Thr | Phe | Asp | Gly | Thr | Pro |     |
|     |     |     | 160 |     |     |     |     | 165 |     |     |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AGC | TAC | GGT | CAC | ACG | CCC | TCG | CAC | CAT | GCG | GCG | CAG | TTC | 546 |
| Ser | Tyr | Gly | His | Thr | Pro | Ser | His | His | Ala | Ala | Gln | Phe |     |
|     | 170 |     |     |     | 175 |     |     |     |     | 180 |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CCC | AAC | CAC | TCA | TTC | AAG | CAT | GAG | GAT | CCG | GCT | GCT | AAC | 585 |
| Pro | Asn | His | Ser | Phe | Lys | His | Glu | Asp | Pro | Ala | Ala | Asn |     |
|     |     | 185 |     |     |     |     | 190 |     |     |     |     | 195 |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AAA | GCC | CGA | AAG | GAA | GCT | GAG | TTG | GCT | GCT | GCC | ACC | GCT | 624 |
| Lys | Ala | Arg | Lys | Glu | Ala | Glu | Leu | Ala | Ala | Ala | Thr | Ala |     |
|     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| GAG | CAA | TAA |     |     |     |     |     |     |     |     |     |     | 633 |
| Glu | Gln |     |     |     |     |     |     |     |     |     |     |     |     |
|     | 210 |     |     |     |     |     |     |     |     |     |     |     |     |

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 210 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Arg | Gly | Ser | His | His | His | His | His | His | Ser | Met | Gly | Ser |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Asp | Val | Arg | Asp | Leu | Asn | Ala | Leu | Leu | Pro | Ala | Val | Pro | Ser |
| 15  |     |     |     |     | 20  |     |     |     |     | 25  |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Leu | Gly | Gly | Gly | Gly | Gly | Cys | Ala | Leu | Pro | Val | Ser | Gly | Ala |
|     | 30  |     |     |     |     | 35  |     |     |     |     | 40  |     |     |

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Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala  
                   45                                  50  55

Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala  
                           60  65  70

Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys  
                                   75  80

Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln  
 85  90  95

Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr  
           100  105  110

Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro  
                   115  120  125

Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro  
                   130  135  140

Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala  
                           145  150

Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr  
 155  160  165

Pro Ser Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe  
           170  175  180

Pro Asn His Ser Phe Lys His Glu Asp Pro Ala Ala Asn Lys  
           185  190  195

Ala Arg Lys Glu Ala Glu Leu Ala Ala Ala Thr Ala Glu Gln  
                   200  205  210

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1680 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 381..1670





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|                   |            |            |            |            |            |            |            |            |            |            |            |            |      |
|-------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------|
| GGG<br>Gly<br>110 | CCC<br>Pro | TTC<br>Phe | GGT<br>Gly | CCT<br>Pro | CCT<br>Pro | CCG<br>Pro | CCC<br>Pro | AGC<br>Ser | CAG<br>Gln | GCG<br>Ala | TCA<br>Ser | TCC<br>Ser | 746  |
|                   |            |            |            |            |            |            | 115        |            |            | 120        |            |            |      |
| GGC<br>Gly        | CAG<br>Gln | GCC<br>Ala | AGG<br>Arg | ATG<br>Met | TTT<br>Phe | CCT<br>Pro | AAC<br>Asn | GCG<br>Ala | CCC<br>Pro | TAC<br>Tyr | CTG<br>Leu | CCC<br>Pro | 785  |
|                   |            | 125        |            |            |            |            | 130        |            |            |            |            | 135        |      |
| AGC<br>Ser        | TGC<br>Cys | CTC<br>Leu | GAG<br>Glu | AGC<br>Ser | CAG<br>Gln | CCC<br>Pro | GCT<br>Ala | ATT<br>Ile | CGC<br>Arg | AAT<br>Asn | CAG<br>Gln | GGT<br>Gly | 824  |
|                   |            |            |            | 140        |            |            |            |            | 145        |            |            |            |      |
| TAC<br>Tyr        | AGC<br>Ser | ACG<br>Thr | GTC<br>Val | ACC<br>Thr | TTT<br>Phe | GAC<br>Asp | GGG<br>Gly | ACG<br>Thr | CCC<br>Pro | AGC<br>Ser | TAC<br>Tyr | GGT<br>Gly | 863  |
|                   | 150        |            |            |            |            | 155        |            |            |            |            | 160        |            |      |
| CAC<br>His        | ACG<br>Thr | CCC<br>Pro | TCG<br>Ser | CAC<br>His | CAT<br>His | GCG<br>Ala | GCG<br>Ala | CAG<br>Gln | TTC<br>Phe | CCC<br>Pro | AAC<br>Asn | CAC<br>His | 902  |
|                   |            |            | 165        |            |            |            |            | 170        |            |            |            |            |      |
| TCA<br>Ser        | TTC<br>Phe | AAG<br>Lys | CAT<br>His | GAG<br>Glu | GAT<br>Asp | CCC<br>Pro | ATG<br>Met | GGC<br>Gly | CAG<br>Gln | CAG<br>Gln | GGC<br>Gly | TCG<br>Ser | 941  |
| 175               |            |            |            |            | 180        |            |            |            |            | 185        |            |            |      |
| CTG<br>Leu        | GGT<br>Gly | GAG<br>Glu | CAG<br>Gln | CAG<br>Gln | TAC<br>Tyr | TCG<br>Ser | GTG<br>Val | CCG<br>Pro | CCC<br>Pro | CCG<br>Pro | GTC<br>Val | TAT<br>Tyr | 980  |
|                   |            | 190        |            |            |            |            | 195        |            |            |            |            | 200        |      |
| GGC<br>Gly        | TGC<br>Cys | CAC<br>His | ACC<br>Thr | CCC<br>Pro | ACC<br>Thr | GAC<br>Asp | AGC<br>Ser | TGC<br>Cys | ACC<br>Thr | GGC<br>Gly | AGC<br>Ser | CAG<br>Gln | 1019 |
|                   |            |            |            | 205        |            |            |            |            | 210        |            |            |            |      |
| GCT<br>Ala        | TTG<br>Leu | CTG<br>Leu | CTG<br>Leu | AGG<br>Arg | ACG<br>Thr | CCC<br>Pro | TAC<br>Tyr | AGC<br>Ser | AGT<br>Ser | GAC<br>Asp | AAT<br>Asn | TTA<br>Leu | 1058 |
|                   | 215        |            |            |            |            | 220        |            |            |            |            | 225        |            |      |
| TAC<br>Tyr        | CAA<br>Gln | ATG<br>Met | ACA<br>Thr | TCC<br>Ser | CAG<br>Gln | CTT<br>Leu | GAA<br>Glu | TGC<br>Cys | ATG<br>Met | ACC<br>Thr | TGG<br>Trp | AAT<br>Asn | 1097 |
|                   |            |            | 230        |            |            |            |            | 235        |            |            |            |            |      |
| CAG<br>Gln        | ATG<br>Met | AAC<br>Asn | TTA<br>Leu | GGA<br>Gly | GCC<br>Ala | ACC<br>Thr | TTA<br>Leu | AAG<br>Lys | GGA<br>Gly | CAC<br>His | AGC<br>Ser | ACA<br>Thr | 1136 |
| 240               |            |            |            |            | 245        |            |            |            |            | 250        |            |            |      |
| GGG<br>Gly        | TAC<br>Tyr | GAG<br>Glu | AGC<br>Ser | GAT<br>Asp | AAC<br>Asn | CAC<br>His | ACA<br>Thr | ACG<br>Thr | CCC<br>Pro | ATC<br>Ile | CTC<br>Leu | TGC<br>Cys | 1175 |
|                   |            | 255        |            |            |            |            | 260        |            |            |            |            | 265        |      |
| GGA<br>Gly        | GCC<br>Ala | CAA<br>Gln | TAC<br>Tyr | AGA<br>Arg | ATA<br>Ile | CAC<br>His | ACG<br>Thr | CAC<br>His | GGT<br>Gly | GTC<br>Val | TTC<br>Phe | AGA<br>Arg | 1214 |
|                   |            |            |            | 270        |            |            |            |            | 275        |            |            |            |      |



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## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 429 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala
 1           5           10
Val Pro Ser Leu Gly Gly Gly Gly Cys Ala Leu Pro Val
15           20           25
Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro
30           35           40
Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro
45           50           55
Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser
60           65           70
Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His
75           80
Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly
85           90           95
Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
100          105          110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg
115          120          125
Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser
130          135          140
Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe
145          150
Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala
155          160          165
Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met
170          175          180
Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro
185          190          195

```

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Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr  
 200 205 210  
 Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp  
 215 220  
 Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp  
 225 230 235  
 Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr  
 240 245 250  
 Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly  
 255 260 265  
 Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile  
 270 275 280  
 Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu Val  
 285 290  
 Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys  
 295 300 305  
 Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His  
 310 315 320  
 Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr  
 325 330 335  
 Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser  
 340 345 350  
 Asp Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys  
 355 360  
 Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser  
 365 370 375  
 Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys  
 380 385 390  
 Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala  
 395 400 405  
 Arg Ser Asp Glu Leu Val Arg His His Asn Met His Gln Arg  
 410 415 420  
 Asn Met Thr Lys Leu Gln Leu Ala Leu  
 425

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGAGAGGA TCGCATCACC ATCACCATCA CTC 33

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATGGAGTGA TGGTGATGGT GATGCGATCC TCT 33

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 17 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Val Ala Ala Gly Ser Ser Ser Ser Val Lys Trp Thr Glu Gly  
 1 5 10

Gln Ser Asn  
 15

## WHAT IS CLAIMED IS:

1. A hybridoma cell line which produces a monoclonal antibody specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4.
2. The hybridoma cell line according to claim 1, wherein said cell line is ATCC No. 11598.
3. The hybridoma cell line according to claim 1, wherein said cell line is ATCC No. 11599.
4. The hybridoma cell line according to claim 1, wherein said cell line is ATCC No. 11560.
5. A monoclonal antibody directed against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4, said antibody capable of specifically binding to Wilms' tumor protein.
6. The antibody according to claim 5, wherein said antibody is H2.
7. The antibody according to claim 5, wherein said antibody is H7.
8. The antibody according to claim 5, wherein said antibody is HC17.
9. A polypeptide derived from a monoclonal antibody directed against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4, said polypeptide selected from the group consisting of
  - (a) heavy chain variable region polypeptides of said monoclonal antibody;

(b) light chain variable region polypeptides of said monoclonal antibody;

(c) a Fab fragment of said antibody;

(d) a F(ab)<sub>2</sub> fragment of said antibody; and

(e) an Fv fragment of said antibody.

10. A method for diagnosing a disease condition characterized by WT1 expression comprising the steps of:

a) providing a biological sample from a patient having the clinical symptoms associated with mesothelioma;

b) contacting said sample with a monoclonal antibody or functional fragment thereof specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4; and

c) detecting the presence of binding of said monoclonal antibody or fragment to said biological sample, wherein the presence of such binding indicates the presence of said disease condition.

11. The method according to claim 10, wherein said antibody is selected from the group consisting of H2, H7, HC17, and a cocktail thereof.

12. The method according to claim 10, wherein said biological sample is selected from the group consisting of whole blood, serum, plasma, synovial fluid, and tissue and said disease condition is selected from the group consisting of mesothelioma, prostate cancer, ovarian cancer, and leukemia.



13. A method of monitoring therapy in leukemia patients comprising the steps of:

a) providing a biological sample from a patient treated for leukemia;

b) contacting said sample with a monoclonal antibody or functional fragment thereof specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4; and

c) detecting the presence of binding of said monoclonal antibody or fragment thereof to said biological sample, wherein the presence of such binding indicates the presence of a active leukemia cells.

14. The method according to claim 13, wherein said antibody is selected from the group consisting of H2, H7, HC17, and a cocktail thereof.

15. The method according to claim 13, wherein said biological sample is selected from the group consisting of whole blood, plasma, serum, urine and bone marrow.

16. The use of a monoclonal antibody raised against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4 in detecting a disease characterized by the expression of the Wilms' tumor antigen.

17. A kit for diagnosing a disease characterized by the expression of the Wilms' tumor antigen comprising a monoclonal antibody raised against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4 and means for signal generation.

18. The kit according to claim 17 wherein said monoclonal antibody is selected from the group consisting of H2, H7 and HC17.

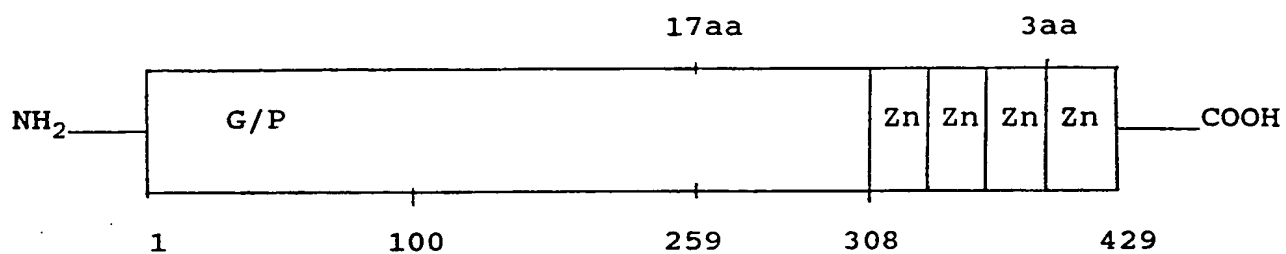
19. An antibody construct comprising at least one complementarity determining region from a monoclonal antibody specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4, said construct selected from the group consisting of a humanized antibody, a chimeric antibody, and a bi-specific antibody.

20. The antibody according to claim 19 wherein said monoclonal antibody is selected from the group consisting of H2, H7 and HC17.

21. A method for producing an antibody construct comprising employing at least one complementarity determining region or heavy chain variable region from a monoclonal antibody specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4.

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FIGURE 1



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FIGURE 2A

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ATG | AGA | GGA | TCG | CAT | CAC | CAT | CAC | CAT | CAC | TCC | ATG | GGT | TCC | 42  |
| Met | Arg | Gly | Ser | His | His | His | His | His | His | Ser | Met | Gly | Ser |     |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     |     |
| GAC | GTT | CGT | GAC | CTG | AAC | GCA | CTG | CTG | CCG | GCA | GTT | CCG | TCC | 84  |
| Asp | Val | Arg | Asp | Leu | Asn | Ala | Leu | Leu | Pro | Ala | Val | Pro | Ser |     |
| 15  |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     |     |
| CTG | GGT | GGT | GGT | GGT | GGT | TGC | GCA | CTG | CCG | GTT | AGC | GGT | GCA | 126 |
| Leu | Gly | Gly | Gly | Gly | Gly | Cys | Ala | Leu | Pro | Val | Ser | Gly | Ala |     |
|     | 30  |     |     |     |     | 35  |     |     |     |     | 40  |     |     |     |
| GCA | CAG | TGG | GCT | CCG | GTT | CTG | GAC | TTC | GCA | CCG | CCG | GGT | GCA | 168 |
| Ala | Gln | Trp | Ala | Pro | Val | Leu | Asp | Phe | Ala | Pro | Pro | Gly | Ala |     |
|     |     | 45  |     |     |     |     | 50  |     |     |     |     | 55  |     |     |
| TCC | GCA | TAC | GGT | TCC | CTG | GGT | GGT | CCG | GCA | CCG | CCG | CCG | GCA | 210 |
| Ser | Ala | Tyr | Gly | Ser | Leu | Gly | Gly | Pro | Ala | Pro | Pro | Pro | Ala |     |
|     |     |     | 60  |     |     |     |     | 65  |     |     |     |     | 70  |     |
| CCG | CCG | CCG | CCG | CCG | CCG | CCG | CCG | CCG | CAC | TCC | TTC | ATC | AAA | 252 |
| Pro | Pro | Pro | Pro | Pro | Pro | Pro | Pro | Pro | His | Ser | Phe | Ile | Lys |     |
|     |     |     |     | 75  |     |     |     |     | 80  |     |     |     |     |     |
| CAG | GAA | CCG | AGC | TGG | GGT | GGT | GCA | GAA | CCG | CAC | GAA | GAA | CAG | 294 |
| Gln | Glu | Pro | Ser | Trp | Gly | Gly | Ala | Glu | Pro | His | Glu | Glu | Gln |     |
| 85  |     |     |     | 90  |     |     |     |     |     | 95  |     |     |     |     |
| TGC | CTG | AGC | GCA | TTC | ACC | GTT | CAC | TTC | TCC | GGC | CAG | TTC | ACT | 336 |
| Cys | Leu | Ser | Ala | Phe | Thr | Val | His | Phe | Ser | Gly | Gln | Phe | Thr |     |
|     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |     |
| GGC | ACA | GCC | GGA | GCC | TGT | CGC | TAC | GGG | CCC | TTC | GGT | CCT | CCT | 378 |
| Gly | Thr | Ala | Gly | Ala | Cys | Arg | Tyr | Gly | Pro | Phe | Gly | Pro | Pro |     |
|     |     | 115 |     |     |     |     | 120 |     |     |     |     | 125 |     |     |
| CCG | CCC | AGC | CAG | GCG | TCA | TCC | GGC | CAG | GCC | AGG | ATG | TTT | CCT | 420 |
| Pro | Pro | Ser | Gln | Ala | Ser | Ser | Gly | Gln | Ala | Arg | Met | Phe | Pro |     |
|     |     |     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |
| AAC | GCG | CCC | TAC | CTG | CCC | AGC | TGC | CTC | GAG | AGC | CAG | CCC | GCT | 462 |
| Asn | Ala | Pro | Tyr | Leu | Pro | Ser | Cys | Leu | Glu | Ser | Gln | Pro | Ala |     |
|     |     |     |     | 145 |     |     |     |     | 150 |     |     |     |     |     |
| ATT | CGC | AAT | CAG | GGT | TAC | AGC | ACG | GTC | ACC | TTC | GAC | GGG | ACG | 504 |
| Ile | Arg | Asn | Gln | Gly | Tyr | Ser | Thr | Val | Thr | Phe | Asp | Gly | Thr |     |
| 155 |     |     |     | 160 |     |     |     |     |     | 165 |     |     |     |     |

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FIGURE 2B

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CCC | AGC | TAC | GGT | CAC | ACG | CCC | TCG | CAC | CAT | GCG | GCG | CAG | TTC | 546 |
| Pro | Ser | Tyr | Gly | His | Thr | Pro | Ser | His | His | Ala | Ala | Gln | Phe |     |
|     | 170 |     |     |     |     | 175 |     |     |     |     | 180 |     |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CCC | AAC | CAC | TCA | TTC | AAG | CAT | GAG | GAT | CCG | GCT | GCT | AAC | AAA | 588 |
| Pro | Asn | His | Ser | Phe | Lys | His | Glu | Asp | Pro | Ala | Ala | Asn | Lys |     |
|     | 185 |     |     |     |     |     | 190 |     |     |     |     | 195 |     |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| GCC | CGA | AAG | GAA | GCT | GAG | TTG | GCT | GCT | GCC | ACC | GCT | GAG | CAA | 630 |
| Ala | Arg | Lys | Glu | Ala | Glu | Leu | Ala | Ala | Ala | Thr | Ala | Glu | Gln |     |
|     |     |     | 200 |     |     |     |     | 205 |     |     |     |     | 210 |     |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TAA |     |     |     |     |     |     |     |     |     |     |     |     |     | 633 |

FIGURE 3A

|   |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|---|------------|------------|---------------------|------------|----|-----|----|----|--|----|-----|-----|----|-----|
| GTTCAAGGCA  | GCGCCACAC  | CCGGGGGCTC | TGCGCAACCC          | GACCGCCTGT |    |     |    |    |  |    |     |     |    | 50  |
| CCGCTCCCC   | ACTTCCCGCC | CTCCCTCCCA | CCTACTCATT          | CACCCACCCA |    |     |    |    |  |    |     |     |    | 100 |
| CCCACCCAGA  | GCCGGGACGG | CAGCCCAGGC | GCCC GG GCC         | CGCCGTCTCC |    |     |    |    |  |    |     |     |    | 150 |
| TCGCCGCGAT  | CCTGGACTTC | CTCTTGCTGC | AGGACCCGGC          | TTCCACGTGT |    |     |    |    |  |    |     |     |    | 200 |
| GTCCCGGAGC  | CGGCGTCTCA | GCACACGCTC | CGCTCCGGGC          | CTGGGTGCCT |    |     |    |    |  |    |     |     |    | 250 |
| ACAGCAGCCA  | GAGCAGCAGG | GAGTCCGGGA | CCCGGGCGGC          | ATCTGGGCCA |    |     |    |    |  |    |     |     |    | 300 |
| AGTTAGGCGC  | CGCCGAGGCC | AGCGCTGAAC | GTCTCCAGGG          | CCGGAGGAGC |    |     |    |    |  |    |     |     |    | 350 |
| CGCGGGGCGT  | CCGGGTCTGA | GCCTCAGCAA | ATG GGC TCC GAC GTG |            |    |     |    |    |  |    |     |     |    | 395 |
|   |            |            | Met Gly Ser Asp Val |            |    |     |    |    |  |    |     |     |    |     |
|   |            |            | 1                   |            |    |     |    |    |  |    |     |     |    | 5   |
| CGG GAC CTG AAC GCG CTG CTG CCC GCC GTC CCC TCC CTG GGT |            |            |                     |            |    |     |    |    |  |    |     |     |    | 437 |
| Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   |            |            |                     | 10         |    |     |    |    |  | 15 |     |     |    |     |
| GGC GGC GGC GGC TGT GCC CTG CCT GTG AGC GGC GCG GCG CAG |            |            |                     |            |    |     |    |    |  |    |     |     |    | 479 |
| Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   | 20         |            |                     |            | 25 |     |    |    |  |    | 30  |     |    |     |
| TGG GCG CCG GTG CTG GAC TTT GCG CCC CCG GGC GCT TCG GCT |            |            |                     |            |    |     |    |    |  |    |     |     |    | 521 |
| Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   | 35         |            |                     |            |    | 40  |    |    |  |    |     | 45  |    |     |
| TAC GGG TCG TTG GGC GGC CCC GCG CCG CCA CCG GCT CCG CCG |            |            |                     |            |    |     |    |    |  |    |     |     |    | 563 |
| Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   |            | 50         |                     |            |    |     | 55 |    |  |    |     |     | 60 |     |
| CCA CCC CCG CCG CCG CCG CCT CAC TCC TTC ATC AAA CAG GAG |            |            |                     |            |    |     |    |    |  |    |     |     |    | 605 |
| Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   |            |            | 65                  |            |    |     |    | 70 |  |    |     |     | 75 |     |
| CCG AGC TGG GGC GGC GCG GAG CCG CAC GAG GAG CAG TGC CTG |            |            |                     |            |    |     |    |    |  |    |     |     |    | 647 |
| Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   |            |            | 80                  |            |    |     |    |    |  | 85 |     |     |    |     |
| AGC GCC TTC ACT GTC CAC TTT TCC GGC CAG TTC ACT GGC ACA |            |            |                     |            |    |     |    |    |  |    |     |     |    | 689 |
| Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   | 90         |            |                     |            | 95 |     |    |    |  |    | 100 |     |    |     |
| GCC GGA GCC TGT CGC TAC GGG CCC TTC GGT CCT CCT CCG CCC |            |            |                     |            |    |     |    |    |  |    |     |     |    | 731 |
| Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro |            |            |                     |            |    |     |    |    |  |    |     |     |    |     |
|   | 105        |            |                     |            |    | 110 |    |    |  |    |     | 115 |    |     |

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FIGURE 3B

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| AGC | CAG | GCG | TCA | TCC | GGC | CAG | GCC | AGG | ATG | TTT | CCT | AAC | GCG | 773  |
| Ser | Gln | Ala | Ser | Ser | Gly | Gln | Ala | Arg | Met | Phe | Pro | Asn | Ala |      |
|     |     | 120 |     |     |     |     | 125 |     |     |     |     | 130 |     |      |
| CCC | TAC | CTG | CCC | AGC | TGC | CTC | GAG | AGC | CAG | CCC | GCT | ATT | CGC | 815  |
| Pro | Tyr | Leu | Pro | Ser | Cys | Leu | Glu | Ser | Gln | Pro | Ala | Ile | Arg |      |
|     |     |     | 135 |     |     |     |     | 140 |     |     |     |     | 145 |      |
| AAT | CAG | GGT | TAC | AGC | ACG | GTC | ACC | TTC | GAC | GGG | ACG | CCC | AGC | 857  |
| Asn | Gln | Gly | Tyr | Ser | Thr | Val | Thr | Phe | Asp | Gly | Thr | Pro | Ser |      |
|     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     |      |
| TAC | GGT | CAC | ACG | CCC | TCG | CAC | CAT | GCG | GCG | CAG | TTC | CCC | AAC | 899  |
| Tyr | Gly | His | Thr | Pro | Ser | His | His | Ala | Ala | Gln | Phe | Pro | Asn |      |
| 160 |     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |      |
| CAC | TCA | TTC | AAG | CAT | GAG | GAT | CCC | ATG | GGC | CAG | CAG | GGC | TCG | 941  |
| His | Ser | Phe | Lys | His | Glu | Asp | Pro | Met | Gly | Gln | Gln | Gly | Ser |      |
|     | 175 |     |     |     |     | 180 |     |     |     |     | 185 |     |     |      |
| CTG | GGT | GAG | CAG | CAG | TAC | TCG | GTG | CCG | CCC | CCG | GTC | TAT | GGC | 983  |
| Leu | Gly | Glu | Gln | Gln | Tyr | Ser | Val | Pro | Pro | Pro | Val | Tyr | Gly |      |
|     |     | 190 |     |     |     |     | 195 |     |     |     |     | 200 |     |      |
| TGC | CAC | ACC | CCC | ACC | GAC | AGC | TGC | ACC | GGC | AGC | CAG | GCT | TTG | 1025 |
| Cys | His | Thr | Pro | Thr | Asp | Ser | Cys | Thr | Gly | Ser | Gln | Ala | Leu |      |
|     |     |     | 205 |     |     |     |     | 210 |     |     |     |     | 215 |      |
| CTG | CTG | AGG | ACG | CCC | TAC | AGC | AGT | GAC | AAT | TTA | TAC | CAA | ATG | 1067 |
| Leu | Leu | Arg | Thr | Pro | Tyr | Ser | Ser | Asp | Asn | Leu | Tyr | Gln | Met |      |
|     |     |     |     | 220 |     |     |     |     | 225 |     |     |     |     |      |
| ACA | TCC | CAG | CTT | GAA | TGC | ATG | ACC | TGG | AAT | CAG | ATG | AAC | TTA | 1109 |
| Thr | Ser | Gln | Leu | Glu | Cys | Met | Thr | Trp | Asn | Gln | Met | Asn | Leu |      |
| 230 |     |     |     |     | 235 |     |     |     |     | 240 |     |     |     |      |
| GGA | GCC | ACC | TTA | AAG | GGA | CAC | AGC | ACA | GGG | TAC | GAG | AGC | GAT | 1151 |
| Gly | Ala | Thr | Leu | Lys | Gly | His | Ser | Thr | Gly | Tyr | Glu | Ser | Asp |      |
|     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |     |      |
| AAC | CAC | ACA | ACG | CCC | ATC | CTC | TGC | GGA | GCC | CAA | TAC | AGA | ATA | 1193 |
| Asn | His | Thr | Thr | Pro | Ile | Leu | Cys | Gly | Ala | Gln | Tyr | Arg | Ile |      |
|     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |      |
| CAC | ACG | CAC | GGT | GTC | TTC | AGA | GGC | ATT | CAG | GAT | GTG | CGA | CGT | 1235 |
| His | Thr | His | Gly | Val | Phe | Arg | Gly | Ile | Gln | Asp | Val | Arg | Arg |      |
|     |     |     | 275 |     |     |     |     | 280 |     |     |     |     | 285 |      |
| GTG | CCT | GGA | GTA | GCC | CCG | ACT | CTT | GTA | CGG | TCG | GCA | TCT | GAG | 1277 |
| Val | Pro | Gly | Val | Ala | Pro | Thr | Leu | Val | Arg | Ser | Ala | Ser | Glu |      |
|     |     |     |     | 290 |     |     |     |     | 295 |     |     |     |     |      |

FIGURE 3C

|     |     |     |     |           |     |     |     |     |     |     |     |     |     |      |
|-----|-----|-----|-----|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| ACC | AGT | GAG | AAA | CGC       | CCC | TTC | ATG | TGT | GCT | TAC | CCA | GGC | TGC | 1319 |
| Thr | Ser | Glu | Lys | Arg       | Pro | Phe | Met | Cys | Ala | Tyr | Pro | Gly | Cys |      |
| 300 |     |     |     |           | 305 |     |     |     |     | 310 |     |     |     |      |
| AAT | AAG | AGA | TAT | TTT       | AAG | CTG | TCC | CAC | TTA | CAG | ATG | CAC | AGC | 1361 |
| Asn | Lys | Arg | Tyr | Phe       | Lys | Leu | Ser | His | Leu | Gln | Met | His | Ser |      |
|     | 315 |     |     |           |     | 320 |     |     |     |     | 325 |     |     |      |
| AGG | AAG | CAC | ACT | GGT       | GAG | AAA | CCA | TAC | CAG | TGT | GAC | TTC | AAG | 1403 |
| Arg | Lys | His | Thr | Gly       | Glu | Lys | Pro | Tyr | Gln | Cys | Asp | Phe | Lys |      |
|     |     | 330 |     |           |     |     | 335 |     |     |     |     | 340 |     |      |
| GAC | TGT | GAA | CGA | AGG       | TTT | TCT | CGT | TCA | GAC | CAG | CTC | AAA | AGA | 1445 |
| Asp | Cys | Glu | Arg | Arg       | Phe | Ser | Arg | Ser | Asp | Gln | Leu | Lys | Arg |      |
|     |     |     | 345 |           |     |     |     | 350 |     |     |     |     | 355 |      |
| CAC | CAA | AGG | AGA | CAT       | ACA | GGT | GTG | AAA | CCA | TTC | CAG | TGT | AAA | 1487 |
| His | Gln | Arg | Arg | His       | Thr | Gly | Val | Lys | Pro | Phe | Gln | Cys | Lys |      |
|     |     |     |     | 360       |     |     |     |     | 365 |     |     |     |     |      |
| ACT | TGT | CAG | CGA | AAG       | TTC | TCC | CGG | TCC | GAC | CAC | CTG | AAG | ACC | 1529 |
| Thr | Cys | Gln | Arg | Lys       | Phe | Ser | Arg | Ser | Asp | His | Leu | Lys | Thr |      |
|     |     |     |     |           | 375 |     |     |     |     | 380 |     |     |     |      |
| CAC | ACC | AGG | ACT | CAT       | ACA | GGT | GAA | AAG | CCC | TTC | AGC | TGT | CGG | 1571 |
| His | Thr | Arg | Thr | His       | Thr | Gly | Glu | Lys | Pro | Phe | Ser | Cys | Arg |      |
|     |     |     |     |           |     | 390 |     |     |     |     | 395 |     |     |      |
| TGG | CCA | AGT | TGT | CAG       | AAA | AAG | TTT | GCC | CGG | TCA | GAT | GAA | TTA | 1613 |
| Trp | Pro | Ser | Cys | Gln       | Lys | Lys | Phe | Ala | Arg | Ser | Asp | Glu | Leu |      |
|     |     | 400 |     |           |     |     | 405 |     |     |     |     | 410 |     |      |
| GTC | CGC | CAT | CAC | AAC       | ATG | CAT | CAG | AGA | AAC | ATG | ACC | AAA | CTC | 1655 |
| Val | Arg | His | His | Asn       | Met | His | Gln | Arg | Asn | Met | Thr | Lys | Leu |      |
|     |     |     | 415 |           |     |     |     | 420 |     |     |     |     | 425 |      |
| CAG | CTG | GCG | CTT | TGAGGGTCT | CCC |     |     |     |     |     |     |     |     | 1680 |
| Gln | Leu | Ala | Leu |           |     |     |     |     |     |     |     |     |     |      |



**MICROORGANISMS**

Optional Sheet in connection with the microorganism referred to on page 3, line 32-33 of the description \*

**A. IDENTIFICATION OF DEPOSIT \***

Further deposits are identified on an additional sheet  \*

Name of depository institution \*

American Type Culture Collection

Address of depository institution (including postal code and country) \*

12301 Parklawn Drive  
Rockville, Maryland 20852 USA

Date of deposit \*

31 March 1994

Accession Number \*

HB 11598

**B. ADDITIONAL INDICATIONS \*** (leave blank if not applicable). This information is continued on a separate attached sheet

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE \*** (If the indications are not for all designated States)

**D. SEPARATE FURNISHING OF INDICATIONS \*** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later \* (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E.  This sheet was received with the international application when filed (to be checked by the receiving Office)

*Virginia L. Liley*  
(Authorized Officer)

The date of receipt (from the applicant) by the International Bureau \*

was

(Authorized Officer)

**MICROORGANISMS**

Optional Sheet in connection with the microorganism referred to on page 3, line 34-35 of the description <sup>1</sup>

**A. IDENTIFICATION OF DEPOSIT <sup>1</sup>**

Further deposits are identified on an additional sheet  <sup>2</sup>

Name of depository institution <sup>3</sup>

American Type Culture Collection

Address of depository institution (including postal code and country) <sup>4</sup>

12301 Parklawn Drive  
Rockville, Maryland 20852  
United States of America

Date of deposit <sup>5</sup>

31 March 1994

Accession Number <sup>6</sup>

HB 11599

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**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE <sup>8</sup>** (if the indications are not for all designated States)

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The indications listed below will be submitted to the International Bureau later <sup>9</sup> (Specify the general nature of the indications e.g., "Accession Number of Deposit")

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**MICROORGANISMS**

Optional Sheet in connection with the microorganism referred to on page 4, line 1-2 of the description 1

**A. IDENTIFICATION OF DEPOSIT 1**

Further deposits are identified on an additional sheet  2

Name of depository institution 4

American Type Culture Collection

Address of depository institution (including postal code and country) 4

12301 Parklawn Drive  
Rockville, Maryland 20852  
United States of America

Date of deposit 4

31 March 1994

Accession Number 4

HB 11600

**B. ADDITIONAL INDICATIONS 1** (leave blank if not applicable). This information is continued on a separate attached sheet

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE 2** (if the indications are not for all designated States)

**D. SEPARATE FURNISHING OF INDICATIONS 3** (leave blank if not applicable)

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*Virginia L. Liley*

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/05523**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 5/12, 15/02; C12P 21/08; G01N 33/574; C07K 16/18, 16/30  
 US CL : 435/240.27, 172.2, 70.21, 7.23; 530/387.9, 387.3, 388.8, 388.85  
 According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.27, 172.2, 70.21, 7.23; 530/387.9, 387.3, 388.8, 388.85

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: CA, BIOSIS, MEDLINE, EMBASE, CANCERLIT, BIOTECHDS, GENESEQ, SWISS-PROT, PIR, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| Y         | ONCOGENE, VOLUME 6, NUMBER 12, ISSUED 12 DECEMBER 1991, MORRIS ET AL, "CHARACTERIZATION OF THE ZINC FINGER PROTEIN ENCODED BY THE WT1 WILMS' TUMOR LOCUS", PAGES 2339-2348, SEE ENTIRE DOCUMENT.  | 1-21                  |
| Y         | PROCEEDINGS NATIONAL ACADEMY OF SCIENCE, VOLUME 90, ISSUED JUNE 1993, MAHESWARAN ET AL, "PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN WT1 AND p53 PROTEINS", PAGES 5100-5104, SEE ENTIRE DOCUMENT. | 1-21                  |
| Y         | WO 91/07509 (CALL ET AL) 30 MAY 1991, SEE ENTIRE DOCUMENT.  | 1-21                  |

 Further documents are listed in the continuation of Box C.
  See patent family annex.

|   |  |
|---|--|
| * Special categories of cited documents:  | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| "A" document defining the general state of the art which is not considered to be of particular relevance  | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| "E" earlier document published on or after the international filing date  | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "G" document member of the same patent family  |
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| "P" document published prior to the international filing date but later than the priority date claimed  |  |

|   |   |
|---|---|
| Date of the actual completion of the international search<br>17 JULY 1995 | Date of mailing of the international search report<br>03 AUG 1995 |
|---|---|

Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SUSAN A. LORING

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/05523

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| Y         | CLINICAL CHEMISTRY, VOLUME 27, NUMBER 11, ISSUED 1981, SEVIER ET AL, "MONOCLONAL ANTIBODIES IN CLINICAL IMMUNOLOGY", PAGES 1797-1806, SEE ENTIRE DOCUMENT.   | 1-21                  |
| Y         | NATURE, VOLUME 351, ISSUED 06 JUNE 1991, CO ET AL, "HUMANIZED ANTIBODIES FOR THERAPY", PAGES 501-502, SEE ENTIRE DOCUMENT.   | 19-21                 |
| Y         | PROCEEDINGS NATIONAL ACADEMY OF SCIENCE, VOLUME 81, ISSUED NOVEMBER 1984, MORRISON ET AL, "CHIMERIC HUMAN ANTIBODY MOLECULES: MOUSE ANTIGEN-BINDING DOMAINS WITH HUMAN CONSTANT REGION DOMAINS", PAGES 6851-6855, SEE ENTIRE DOCUMENT. | 19-21                 |
| Y         | US,A,5,141,736 (IWASA ET AL) 25 AUGUST 1992, SEE ENTIRE DOCUMENT.  | 19-21                 |

